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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 28 February 2000 (28.02.00)	
International application No. PCT/NZ99/00107	**Applicant's or agent's file reference** P408128 CXE
International filing date (day/month/year) 07 July 1999 (07.07.99)	**Priority date** (day/month/year) 07 July 1998 (07.07.98)
Applicant BASS, James, J. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 February 2000 (04.02.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia RANAIVOJAONA

PATENT COOPERATION TREATY

REC'D 13 MAR 2000
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P408128CXE	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International application No. **PCT/NZ99/00107**	International filing date *(day/month/year)* 7 July 1999	Priority Date *(day/month/year)* 7 July 1998
International Patent Classification (IPC) or national classification and IPC **Int. Cl. [7] C12N 015/11**		
Applicant NEW ZEALAND PASTORAL AGRICULTURE RESEARCH INSTITUTE LIMITED		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer GILLIAN ALLEN Telephone No. (02) 6283 2266

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/NZ99/00107

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of .

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-24	**YES**
	Claims		**NO**
Inventive step (IS)	Claims	1-24	**YES**
	Claims		**NO**
Industrial applicability (IA)	Claims	1-24	**YES**
	Claims		**NO**

2. Citations and explanations (Rule 70.7)

Citations

D1 EMBL Acc No AF093798. Sus scrofa myostatin (GDF8) gene, promoter region and partial cds.

D2 McPherson AC and Lee S-J. Double muscling in cattle due to mutations in the myostatin gene.

Novelty

The closest art is that of D1 which discloses the pig myostatin promoter. However this has a publication date later than the priority date of the present claims, and as such, does not constitute a prior art document, or anticipate the present claims

Inventive Step

D2 discloses the sequence of the bovine myostatin gene. However it discloses only the coding region, and provides no promoter sequence.

It would be obvious to one skilled in the art that promoter sequences could be obtained from genomic DNA upstream of the coding region, and is noted that the applicants were able to obtain the genomic material from a commercially available gene library (Stratagene). DNA sequencing is a routine technique of the art.

However, D1 provides no teaching or suggestion to find and sequence the myostatin promoter. Also applicant has, to some extent, characterised protein binding sites within the promoter region, which could not have been predicted from knowledge of the prior art. The claims are therefore accepted as being inventive.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1. Claim 1 is not clear as the term "substantially equivalent function" is undefined. The function of the myostatin promoter is to promote transcription of the myostatin gene. However, a large number of totally heterologous promoters would have this function. To keep the claim within the limits of the disclosures of the description, the term would have to include the tissue specific properties hypothesised for the myostatin promoter of the present invention (p 3: 25-26).

2. Claims 16-18 are not clear. I cannot determine whether, in the phrase "as herein described or exemplified with reference to the accompanying drawings", the qualification that it be described in the drawings refers to only "exemplified" or "described and exemplified". Further, the drawings are DNA sequences, and do not show vectors, probes, DNA constructs or methods of cloning.

3. Claim 3 a) is not clear. The term "at least 70-95% identical" is indeterminate. A range cannot provide a minimum cut off point

4. The claims are not fully supported by the disclosures of the description. Applicants have sequenced the bovine myostatin promoter, and shown that the 3.3kb of sequence upstream of the coding region can promote transcription of the CAT gene in myoblasts. However there is no support, except in general predictions, for the variants, fragments, substituents and homologues encompassed by the claims.
   It is considered reasonable to claim the bovine myostatin promoter, allelic variants, and close myostatin promoter homologues from other species, and DNA constructs that incorporate these sequences. It is also acceptable to claim DNA sequences hybridising under stringent conditions to the disclosed sequence that have use as probes.

Therefore, with the possible exception of the omnibus claims 16-24, all claims lack full support from the description.

NOVEL PROMOTER SEQUENCES OF MYOSTATIN GENE

The present invention concerns the novel promoter sequences of the myostatin gene, DNA constructs comprising the novel promoter sequences operably linked to the coding sequence of a gene of interest, to vectors containing the promoter or construct and host cells containing such vectors.

Background of the Invention

A promoter region of a gene is one which controls the expression of the gene. The myostatin gene was isolated and sequenced and the sequence published (McPherron AC; Lawler AM and Lee SJ; Nature **387**, 1 May 1997; WO94/21681). However the sequence of the promoter region of this gene has not been isolated, sequenced or published to date.

Myostatin, also known as growth and differentiating factor-8 (GDF-8), is structurally related to the transforming growth factor β (TGF-β) superfamily (McPherron *et al* 1997). The myostatin gene has been shown to inhibit muscle growth and initial research concluded that targeted disruption of the myostatin gene in mice leads to a twofold increase in muscle mass (McPherron *et al* 1997) indicating that the myostatin gene is a negative regulator of muscle mass.

Three of the present inventors (Kambadur, Sharma and Bass) have identified a mutation of the myostatin gene in "double muscled" Belgian Blue cattle comprising an 11bp deletion (Kambadur *et al* 1997). This mutation negates the effect of myostatin and leads to a 30% or 40% increase in muscle mass.

The inventors have further observed two Belgian Blue sire lines which possess the same Belgian blue allele, i.e. the 11bp deletion, but which is being expressed in very small amounts. On a northern blot, two bands were seen suggesting that in these animals there is a double mutation of the myostatin gene, ie hypomorphic alleles. This strongly suggests that a failure in the upstream regions of the myostatin gene have resulted in a lack of expression of myostatin and as a consequence increased muscle growth.

Summary of the Invention

The present invention provides an isolated DNA molecule having a polynucleotide sequence of SEQ ID NO. 1 of Figure 1 and which encodes the promoter region of the

bovine myostatin gene, or a fragment or variant of said polynucleotide sequence which has been modified by the insertion, substitution or deletion of one or more nucleotides having substantially equivalent function thereto.

In a further aspect, the present invention provides an isolated DNA construct comprising the DNA molecule of the invention operably linked to a heterologous gene of interest such that the heterologous gene is under transcriptional control of the DNA molecule of the invention.

The DNA molecule may have been isolated from a natural source or may comprise cDNA.

In a further aspect the invention provides recombinant expression vectors which contain a DNA molecule of the invention as defined above, and/or the DNA construct of the invention, and hosts transformed with such vectors and capable of induction of the DNA molecule and/or of inducible expression of the heterologous gene of the DNA construct. In a further aspect the invention provides a method of producing the sequence encoded by the DNA molecule comprising culturing a host cell, transfecting said host cell with a vector containing the DNA molecule of the invention or a fragment or variant thereof and cloning the DNA sequence by known methods.

In still a further aspect, the invention may be said to consist in a method of diagnosing muscle cell disorders in an animal, including a human, using the myostatin gene promoter sequence as defined above, as a diagnostic probe. The diagnostic method may comprise the steps:

i) obtaining a tissue or blood sample from an animal or human;

ii) isolating the DNA by known methods;

iii) optionally isolating myostatin DNA;

iv) probing said DNA with a probe complementary to the myostatin gene promoter sequence;

v) optionally amplifying the amount of myostatin promoter DNA using PCR technology;

vi) analysing the myostatin promoter sequence DNA obtained with the probe for any mutations which may result in muscle cell disorders; and

vii) diagnosing muscle cell disorders caused by myostatin gene promoter sequence anomalies.

The probe may comprise a complementary sequence to a part or the whole of the nucleotide sequences of myostatin promoter DNA of SEQ ID NO. 1.

The probe is preferable genomic or cDNA.

In still a further aspect, the invention consists in a method of selection of animal breeds that express low levels of myostatin. This method comprises the steps:

i) obtaining a tissue or blood sample from an animal or human;

ii) isolating the RNA by known methods;

iii) determining the transcript level (mRNA) of myostatin gene, using Northern blot or RT-PCR technology or RNase Protection Assay.

Preferably the animal is a cow, bull, sheep, pig, human or any other mammal, poultry, fish or any other economically important livestock breed.

In still a further aspect, the invention consists in a method of expressing a heterologous gene of interest specifically in muscle cells comprising the steps:

i) isolating the coding sequence of said heterologous gene;

ii) ligating said coding sequence of said heterologous gene to the myostatin promoter sequence so that said coding sequence is under the transcriptional control of said myostatin promoter;

iii) inserting the ligated construct into a suitable expression vector;

iv) introducing the expression vector into a muscle host cell; and

v) optionally measuring the expression of the heterologous gene by recovering the product.

The method may be carried out *in vitro* in muscle cells in culture or *in vivo* in an animal including a human.

In a still further aspect, the present invention provides a method of producing a heterologous polypeptide or peptide comprising the steps of:

(a) culturing a host cell which has been transformed or transfected with a vector containing the DNA construct as defined above to express the heterologous polypeptide or peptide encoded by the heterologous gene; and optionally

(b) recovering the expressed polypeptide or peptide.

In a still further aspect, the present invention provides a non-human transgenic mammal that expresses a heterologous polypeptide or peptide in their muscle cells, said non-human mammals having been transfected with the DNA constructs of the present invention.

The heterologous gene of interest may be selected from the group consisting of:

a) Myogenic regulatory factors;

b) Myostatin and Myostatin receptor;

c) Oncogenes;

d) Genes that regulate muscle growth or differentiation;

e) Muscular dystrophy gene; and

f) Any genes expressed in muscle.

The expression vector may be selected from the group consisting of Eukaryotic vectors, retroviral vectors or any vectors that are used for gene therapy.

The muscle host cell may be selected from *in vitro* cell culture lines selected from the group consisting of any primary culture of myoblasts or transformed myoblasts, satellite cells or any cell culture where myostatin promoter is active. Alternatively the host muscle cell may comprise a skeletal muscle cell *in vivo* in a host animal.

The host animal may be selected from the group comprising cow, bull, sheep, pig, horse, rat, mouse, poultry, fish or human.

In a still further embodiment the present invention provides a method of expressing myostatin or antisense to myostatin or ribozymes or any foreign gene in a host muscle cell/ myoblast comprising the steps: preparing a construct comprising the myostatin promoter ligated to a coding sequence of interest, so that said coding sequence is under transcriptional control of the myostatin promoter, cloning said construct into gene therapy vectors by standard cloning procedure and transfecting them into a desired cell line or tissue of a live animal or human.

Preferably the host muscle cell is selected from the group consisting of a skeletal muscle cell, a somite, a myoblast or any mesodermally derived cells.

In a still further aspect the present invention provides a method of expressing dominant negative forms of myostatin or of any gene of interest in a host muscle cell comprising the steps: cloning the myostatin promoter ligated to desired mutated or wild type gene wherein said gene is under the transcriptional control of said myostatin promoter, into gene therapy vectors by standard cloning procedures and transfecting them into a desired cell line or tissue of a live animal or human.

Preferably the host muscle cell is selected from a skeletal muscle cell, a somite, a myoblast or myotube.

The method may be carried out *in vitro* in the host muscle cell in culture or *in vivo* in a host animal. The host animal may be selected from the group consisting of cow, bull, sheep, pig, horse, rat, mouse, human, poultry or fish.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to

which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

Description of Figures

One preferred form of the present invention will now be described with reference to the accompanying drawing in which:

Figure 1 shows the promoter sequence of the myostatin gene isolated from bovine muscle (SEQ ID NO. 1) - indicating consensus sequences for basic functional elements of known transcription factor binding sites.

Figure 2 shows the promoter activity of the promoter sequence of Figure 1.

Definitions

The term "isolated" means substantially separated or purified away from contaminating sequences in the cell or organism in which the nucleic acid naturally occurs and includes nucleic acids purified by standard purification techniques as well as nucleic acids prepared by recombinant technology and those chemically synthesised.

The term "variant" as used herein refers to a DNA molecule wherein the nucleotide sequence is substantially identical to the nucleotide sequence set out in Figure 1. The variant may be arrived at by modification of the nucleotide sequence of the DNA molecule by such modifications as insertion, substitution or deletion of one or more nucleic acids, such modifications comprising neutral mutations which do not affect the functioning of the DNA molecule.

The term "substantial sequence identity" means that two nucleotide sequences, when optimally aligned, such as by the programs Clustal W using default gap weights, share at least 60 per cent sequence identity, preferably at least 80 per cent sequence identity, more preferably at least 90 per cent sequence identity and most preferably at least 95 percent sequence identity or more.

The term "DNA construct" means a construct incorporating the nucleic acid molecule of the present invention, or a fractional fragment, neutral mutation or homolog thereof in a postion whereby a heterologous coding sequence is under the control of and operably linked to the promoter sequence of the invention and is capable of expression in a host cell.

The term "neutral mutation" means a mutation, ie a change in the nucleotide or polypeptide sequence such as by deletion, substitution, inversion or insertion, which have no effect on the function of the encoded promoter sequence.

A fragment of a nucleic acid molecule according to the present invention is a portion of the nucleic acid that is less than full length and comprises at least a minimum length capable of hybridising specifically with a nucleic acid molecule according to the present invention (or a sequence complementary thereto) under stringent conditions as defined below. A fragment according to the present invention has at least one of the biological activities of the nucleic acid or polypeptide of the present invention.

Nucleic acid probes and primers can be prepared based on nucleic acids according to the present invention eg the sequence of SEQ ID NO: 1 or 2. A "probe" comprises an isolated nucleic acid attached to a detectable label or reporter molecule well known in the art. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes.

"Primers" are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, preferably a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, eg by the polymerase chain reaction (PCR) or other nucleic acid amplification methods well known in the art. PCR-primer pairs can be derived from the sequence of a nucleic acid according to the present invention, for example, by using computer programs intended for that purpose such as Primer (Version 0.5© 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Methods for preparing and using probes and primers are described, for example, in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed, vol. 1-3, ed Sambrook et al. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, 1989.

Probes or primers can be free in solution or covalently or noncovalently attached to a solid support by standard means.

The term "operably linked" means a first nucleic acid sequence linked to a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter sequence of the present invention is operably linked to a coding sequence of a heterologous gene if the promoter affects the transcription or expression of the coding sequence.

A "recombinant" nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, eg, by genetic engineering techniques.

Techniques for nucleic acid manipulation are described generally in, for example, Sambrook et al. (1989).

The term "stringent conditions" is functionally defined with regard to the hybridization of a nucleic acid probe to a target nucleic acid (ie to a particular nucleic acid sequence of interest) by the hybridization procedure discussed in Sambrook et al. (1989) at 9.52-9.55 and 9.56-9.58.

Regarding the amplification of a target nucleic acid sequence (eg by PCR) using a particular amplification primer pair, stringent conditions are conditions that permit the primer pair to hybridize only to the target nucleic acid sequence to which a primer having the corresponding wild type sequence (or its complement) would bind.

Nucleic acid hybridization is affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art.

Detailed Description of the Invention

As defined above, in its primary aspect, the present invention is directed to a novel promoter sequence of the myostatin gene and the uses thereof as a diagnostic test for

muscle cell disorders and genetic marker for the selection, in particular, of cattle/sheep breeds that express low levels of myostatin. The novel promoter sequence is also useful for expressing foreign genes or dominant negative forms of myostatin in muscle.

The novel promoter sequence of the present invention comprises an isolated DNA molecule having a polynucleotide sequence of SEQ ID NO: 1 of Figure 1 or a fragment or variant thereof having substantially equivalent transcriptional activity there.

The technology used to isolate the myostatin promoter from a genomic DNA library is described generally in Sambrook et al, "Molecular cloning", Second Edition, Cold Spring Harbour Press (1987).

An initial step involves the use of a specific myostatin DNA probe followed by cloning in a host cell and subsequent amplification using PCR technology.

Preferably, the host cell in which the DNA sequence encoding the myostatin promoter is cloned is a prokaryote such as *E. coli*. Other prokaryotes can also be used, for example bacilli such as *Bacillus subtilis* and enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*.

In general, where the host cell is a prokaryote, cloning vectors containing replication and control sequences which are derived from species compatible with the host cell are used. The vector may also carry marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* has commonly been transformed using pBR322, a plasmid derived from an *E. coli* species which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.

In addition to prokaryotes, eukaryotic microbes, such as yeast may also be used, for example, *Saccharomyces cerevisiae* or cultured cells derived from multicellular organisms such as mammals and insects may be used as hosts. Examples of mammalian cultured cells which may be useful are HeLa cells and Chinese Hamster Ovary (CHO) cells, myoblasts, muscle cells, fibroblasts, and satellite cells.

Suitable cloning vectors for use in mammalian cells include SV40 or other suitable viral vectors.

The DNA molecule of the invention can be contained within a DNA molecule isolated from an appropriate natural source (muscle, blood) or can be produced as intron-free cDNA using conventional techniques. cDNA is preferred.

The invention also contemplates variants of the DNA molecule which differ from the native sequence by the insertion, substitution or deletion of one or more nucleotides. These variants can be made through selective synthesis of the DNA using an appropriate synthesizer or by modification of native DNA by, for example, site specific or cassette mutagenesis or by any other techniques known in the art. Such variants may be between 60-95% homologous in the DNA sequence of SEQ ID NO: 1 of Figure 1 and have substantially equivalent function thereto.

The production of DNA fragments, probes and primers is also well within the capabilities of the skilled worker.

The DNA molecule may comprise a native bovine myostatin isolated from any appropriate natural source or can be produced in the form of a synthetic oligonucleotide where the size of the active fragment to be produced permits. By way of example, the Triester method of Matteucci et al, J. Am. Chem. Soc. Vol 103: 3185-3191 (1981) may be employed.

Once obtained, the DNA molecule is treated to be suitable for insertion together with the selected control sequence into the appropriate cloning vector. To this end the DNA is cleaved, tailored and religated as required.

Cleavage is performed by treating with restriction enzyme(s) in a suitable buffer. Any of the large number of commercially available restriction enzymes can be used as specified by the manufacturer. After cleavage, the nucleic acid is recovered by, for example, precipitation with ethanol.

Tailoring of the cleaved DNA is performed using conventional techniques. For example, if blunt ends are required, the DNA may be treated with DNA polymerase I (Klenow), phenol and chloroform extracted and precipitated by ethanol.

Re-ligation can be performed by providing approximately equimolar amounts of the desired components, appropriately tailored for correct matching and treatment with appropriate ligase (eg T4 DNA ligase).

In a further aspect, the present invention consists in a DNA construct comprising the DNA molecule of the invention, ie the bovine myostatin promoter, operably linked to a heterologous gene of interest such that the heterologous gene is under the transcriptional control of the bovine myostatin promoter.

The constructs may comprise a bovine myostatin promoter operably linked to a heterologous gene of interest selected from the group comprising myogenic regulatory factors; myostatin and myostatin receptor; oncogenes; genes that regulate muscle growth and differentiation; the muscular dystrophy gene; and any other genes expressed in muscle.

Similar techniques to those described above would be employed when making constructs comprising the myostatin promoter ligated to a foreign ie, heterologous gene which may be desired to be expressed in muscle cells either *in vitro* or *in vivo*.

In vitro expression may occur in cultured muscle cells such as fibroblasts, HeLa cells or in cultured myoblasts or myotube cells using replicable transfer vectors suitable for use in the expression of the DNA constructs of the invention to produce a heterologous protein.

The replicable transfer vector may be selected according to the host or host cell to be used. Useful vectors will generally have the following characteristics:

(a) the ability to self-replicate;
(b) the possession of a single target or any particular restriction endonuclease cleavage site; and
(c) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of vectors possessing these characteristics are plasmids and bacterial viruses (bacterophages or phages).

Suitable eukaryotic expression vectors which may be employed include, for example pcDNA 1.1, and the expression product could be isolated and measured.

In vivo expression of a construct of the invention could be brought about by known gene therapy techniques. For example the myostatin promoter could be inserted in the Moloney murine leukemia virus (MoMuLv), Harvey murine sarcoma virus (HaMuSv), murine

mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. By inserting the myostatin promoter into viral vectors, the vector could now be used for expressing muscle specific genes.

In the construction of a vector it is an advantage to be able to distinguish the vector incorporating the foreign DNA from unmodified vectors by a convenient and rapid assay. Reporter systems useful in such assays include reporter genes, and other detectable labels which produce measurable colour changes, antibiotic resistance and the like. In one preferred vector, the β-galactosidase reporter gene is used, which gene is detectable by clones exhibiting a blue phenotype on X-gal plates. This facilitates selection. In one embodiment, the β-galactosidase gene may be replaced by a polyhedron-encoding gene; which gene is detectable by clones exhibiting a white phenotype when stained with X-gal. This blue-white colour selection can serve as a useful marker for detecting recombinant vectors.

Once selected, the vectors may be isolated from the culture using routine procedures such as freeze-thaw extraction followed by purification.

In a still further aspect, the invention provides a method of selecting animals which have low myostatin expression which includes essentially the use of the myostatin promoter sequence as a genetic marker. This could be accomplished by PCR amplification of the myostatin promoter using genomic DNA isolated from blood with the primers designed along the promoter sequence and scanning the genomic DNA for mutations.

Non-limiting examples illustrating the invention will now be provided.

It will be appreciated that the above description is provided by way of example only and variations in both the materials and techniques used which are known to those persons skilled in the art are contemplated.

Example 1

1. Cloning of myostatin gene promoter

5'upstream regulatory sequences of bovine myostatin gene was isolated from a bovine lambda genomic library (Stratagene) by the method of Sambrook et al (1989). The library

was screened with a 500 bp genomic DNA (-1700 to -1200) isolated by inverse PCR. The entire genomic clone was subcloned into pBluescript and sequenced.

This PI-PCR procedure is a modification of the method described by K M Pang and D A Knecht (1997). A series of Sau3A1 digests were prepared for use as templates for PCR. Sau3A1 (New England Biolabs, USA) digests of 10-µg aliquots of bovine genomic DNA were carried out at 2, 0.5, 0.125, 0.031, 0.008, 0.004, 0.002 U/µg DNA for 1 hour at 37°C. The reactions were stopped by heating at 65°C for 20 min. Two micrograms of each reaction mixture were separated on a 1% agarose gel to check the extent of cutting. On the basis of extent of digestion, one partial digest (0.008 U of Sau3A1/µg DNA) was chosen for further work. The DNA was then ethanol precipitated and resuspended in 1x ligase buffer to the final concentration of 5ng/µl. T4 DNA ligase (New England Biolabs, USA) was added to a concentration of 4U/µl to carry out ligation at 22°C overnight. Ligation was then stopped by heating at 65°C for 20 min, phenol and then chloroform-extracted, ethanol precipitated and resuspended in sterile water to a concentration of 50 ng/µl. Two primers (5'- CTGCTCGCTGTTCTCATTCAGATC and 5'-ATCCTCAGTAAACTTCGCCTGGA) facing outwards from 5' translated DNA sequence of bovine myostatin were used to carry out PI-PCR. PCR was carried out in 50 µl reaction volume containing 250 ng re-ligated DNA, 0.2 mM each dNTP (Gibco BRL, USA). 0.8 µM of each primer and 2 µl of Elongase enzyme mix (Gibco BRL, USA) in 1 x "B" buffer supplied by the manufacturer was added. PCR was performed for 35 cycles at: 94°C for 30 sec, 55°C for 2 min, 68°C for 6 min. PCR product was separated on a 1% agarose gel and a 2-kb band was excised, purified with the Wizard PCR Preps DNA Purification System (Promega, USA) and cloned by the TA Cloning System (Invitrogen, USA). 2-kb insert was then sequenced using M13 forward, reverse, 5'-GGCTGTATGTGACATGCG and 5'-TGAACCACTGCACTCTCTTG primers.

2. Construction of CAT reporter vector:

3.3 kb of myostatin upstream genomic DNA was PCR amplified using bovine genomic DNA as template. The primers used in the PCR are as follows GGGGTACCCCAATTCCTGGGACAAATTCTCTA (FORWARD) GGGGTACCGGTTTTAAAATCAATACAATCT (REVERSE). The PCR (35 Cycles) conditions were 94°C for 20 Sec, 50°C for 30 Sec, and 72°C for 60 Sec. The amplified 3.3 kb DNA was cloned into p CAT3 Promoter vector (Promega) as a Kpn I fragment using standard cloning procedures. This construct will be referred to as pMS 26.

3. Transfections and CAT ELISA

Four micrograms of CMV-CAT (positive control), p CAT promoter (negative control) and pMS 26 vectors were transfected into actively growing C2C12 myoblast cultures using Fugene 6 (Roche Diagnostics) according to the manufacturers protocol. Twenty four hours after the transfection the C2C12 cells were made to differentiate in low serum medium for a period of forty eight hours. The cells were subsequently washed and amount of CAT protein in the cell extract was measured using a CAT ELISA kit (Roche Diagnostics) according to the manufacturer's protocol.

RESULTS

3.3 kb of upstream sequences harbours myostatin enhancer sequences

In order to identify the enhancer sequences that drive myostatin expression in a myogenic cell, a reporter CAT construct was made with the 3.3 kb of upstream genomic DNA (pMS 26) and transfected into C2C12 myoblast cell line. The pCAT promoter vector and CMV CAT constructs were used as negative and positive controls respectively in this assay. When cell lysate from transfected C2C12 myoblasts were assayed for CAT protein by ELISA, it was observed that the cells transfected with myostatin promoter construct (pMS 26) had a CAT content of 250 picograms/ml (Fig 2). Whereas in the cells transfected with a negative control vector (p CAT promoter vector) CAT protein content was 5 picograms/ml (Fig 2).

Discussion

Transcriptional regulation plays a crucial role in determining patterns of tissue specific gene expression during development and differentiation. During the early embryonic myogenesis, several transcription factors like MRFs (MyoD, Myogenin, Myf-5 and MRF-5), Pax-3 control the determination, proliferation and the differentiation of myogenic precursor cells (myoblasts) by positively regulating the transcription of muscle specific genes (Rudnicki and Jaenisch, 1995). *Myostatin*, a gene which is shown to negatively regulate the growth of muscle, is expressed early in embryonic myogenesis in somites and the expression is continued in adult axial and paraxial muscle (Kambadur et al., 1997). Furthermore, it is also shown that myostatin gene expression is differentially regulated in different axial and paraxial muscle (Kambadur et al., 1997). Here, we have isolated the upstream sequence of myostatin gene and have characterised the several muscle specific

DNA binding sequences in the upstream genomic DNA. Furthermore, we have also shown that 3.3 kb of upstream DNA can drive the expression of CAT gene in a myogenic precursor cells lines. The isolated upstream sequence has consensus sequences for a typical eukaryotic basal promoter comprising 'CAT' Box and 'TATA' box sequence (Cohen et al 1986, Wingender 1988). A consensus for CAT box (CAAATG) is centered at -206 bp where as consensuses for two TATA boxes are centered at -139 and -163 bp respectively which binds the well characterised basal transcription factor TBP (TATA box Binding Protein), an early step in the formation of pre-initiation complex (Kambadur et al., 1990) of RNA polymerase II mediated transcription. In addition, we have also discovered several 'E' boxes in the upstream sequences of myostatin (Lassar et al 1989). E box has been shown to bind to the basic helix loop helix (bHLH) transcription factors, myogenic regulatory factors like MyoD, Myf-5, MRF-4 and myogenin. These bHLH transcription factors bind to the promoters of several muscle specific genes through bHLH DNA binding domain and control the transcriptional activation of these genes (Rudnicki and Jaenisch, 1995). We have also found a binding site for another muscle specific transcriptional activator MEF-2 (CTAAAAATAAT) at -584 bps in myostatin promoter indicating that myostatin gene regulation may occur by several independent muscle specific transcription factors (Gossett et al 1989).

Conclusion

- A genomic clone containing 10.0 kb of upstream regulatory sequence of *myostatin* is isolated for the first time.
- The enhancer sequences of the myostatin gene have been characterised.
- Several E boxes and one MEF-3 binding site that are specifically observed in muscle specific enhancer sequences are also seen in myostatin enhancer sequences.

It will be appreciated that it is not intended to limit the invention to the aforementioned examples only, many variations, such as might readily occur to a person skilled in the art, being possible without departure from the scope thereof as defined in the accompanying claims.

References

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[3] Lassar B.A. et al (1989) Cell **58**, 823-831.

[4] Gossett L.A. et al (1989) Mol. Cell. Biol. **9**, 5022-5033.

[5] Pang K.M. and Knecht D.A. (1977) Biotechnologies 22:1046-1048

[6] Rudnicki M.A. and Jaenisch R. (1995). BioEssays. **17** 3 203-209

[7] Kambadur R. et al. (1990) Proc. Natl. Acad. Sci **87** 9168-9172

[8] Kambadur R et al (1997) Genome Research 7: 910-915

All of the above listed documents are incorporated into the present specification in their entirety by reference.

CLAIMS:

1. An isolated DNA molecule comprising the polynucleotide sequence of SEQ ID NO: 1 and which encodes the promoter region of the myostatin gene, or a fragment thereof, or variant thereof which has been modified by the insertion, substitution or deletion of one or more nucleotides, said fragment and variant of said polynucleotide sequence having substantially equivalent function thereto.

2. An isolated DNA molecule encoding a promoter region of the myostatin gene, said DNA molecule being selected from the group consisting of:

   a) a DNA molecule that is at least 60% identical to a DNA molecule of SEQ ID NO: 1; and

   b) a DNA molecule that hybridises under standard conditions to a DNA molecule of a).

3. An isolated DNA molecule as claimed in claim 2, selected from the group consisting of:

   a) a DNA molecule that is at least 70-95% identical to a DNA molecule of SEQ ID NO: 1; and

   b) a DNA molecule that hybridises under stringent conditions to a DNA molecule of a).

4. An isolated promoter sequence comprising of at least a portion of the polynucleotide sequence of SEQ ID NO: 1 sufficient to drive expression of a heterologous gene operably linked thereto, or a variant thereof having substantially equivalent function thereto.

5. An isolated promoter sequence as claimed in claim 4, wherein said promoter is tissue specific.

6. An isolated promoter sequence as claimed in claim 5, wherein said promoter is specific for driving expression in muscle cells.

7. An isolated promoter sequence as claimed in any one of claims 4-6 comprising the bovine myostatin promoter or a fragment or variant thereof.

8. A recombinant cloning vector comprising the DNA molecule as claimed in any one of claims 1-3.

9. A host cell transformed or transfected with the recombinant cloning vector as claimed in claim 8.

10. An isolated probe comprising at least 12 consecutive nucleotides of the polynucleotide sequence of SEQ ID NO: 1, or the complement thereof.

11. A recombinant DNA construct comprising the promoter sequence of any one of claims 4-7 operably linked to a coding sequence of a gene of interest.

12. A recombinant DNA construct as claimed in claim 11 wherein the gene of interest is selected from the group consisting of myogenic regulatory factors, myostatin and myostatin receptors, oncogenes, genes that regulate muscle growth and differentiation, muscular dystrophy, and any other gene expressed in muscle.

13. A vector containing the DNA construct as claimed in claim 11 or 12.

14. A host cell transformed or transfected with the vector as claimed in claim 13.

15. A method of cloning the DNA molecule as claimed in any one of claims 1-3 comprising the steps:

a) inserting the isolated DNA molecule into a suitable replicable cloning vector;

b) transforming or transfecting a host cell with said vector *in vitro;*

c) culturing host cells; and

d) isolating cloned DNA molecule.

16. An isolated DNA molecule as claimed in claim 1 substantially as herein described or exemplified with reference to the accompanying drawings.

17. An isolated promoter sequence as claimed in claim 4 substantially as herein described or exemplified with reference to the accompanying drawings.

18. A recombinant cloning vector as claimed in claim 8 substantially as herein described or exemplified with reference to the accompanying drawings.

19. A host cell as claimed in claim 9 substantially as herein described or exemplified with reference to the accompanying drawings.

20. An isolated probe as claimed in claim 10 substantially as herein described or exemplified with reference to the accompanying drawings.

21. A DNA construct as claimed in claim 11 substantially as herein described or exemplified with reference to the accompanying drawings.

22. A vector as claimed in claim 13 substantially as herein described or exemplified with reference to the accompanying drawings.

23. A host cell as claimed in claim 14 substantially as herein described or exemplified with reference to the accompanying drawings.

24. A method as claimed in claim 15 substantially as herein described or exemplified with reference to the accompanying drawings.

SEQ ID NO: 1

GAATTCTTCTATGGCTATCACCATGTCCA -10374

AAACACTACCTGAGTAAAGAAGGAGTCATTTTTTTTTTAAGGGTTTAAAA -10324

AATTTAAAAATTTTTAAATTAAATTTAAAATTGAAATCTAAAAAAATTTT -10274

AAGAAAAATTTTTTTTAATCTGCAGAAACATACTCTGTATTGAGTCTATG -10224

GTTACCAAAGATCCCCCCAGAACAAAACCCCAAGAATTGCAAGATTTTCT -10174

CTTGGGAAGTGTTTGTTTACTGGAAGGCTTATTAACTCAATAATAAGGGA -10124

GAGTAAAGACTTCAAAACTAAAGATCTGTTTTTGTCAGTTCAATCTATAT -10074

TGTCATCTGCTCTGGAAACCCTGAGCTTGTTCTAAAGTAAACTGAACTAT -10024

CATGAAGAAAATCAGCCATCAAAATAGTGAAAACGAACCTCTTACACTCA -9974

GTTTTTTCTCATTTGTAAATTTAAAAAATTTAACTTCATTAAGTCTGGGG -9924

ACCAATGTATAGCTGCAAGCAGGAATTTTATGCCTTCTTAATCCAGAGAG -9874

GTGGGCAATGGTGTGGGTTTAGACCAGAAGTAAGGATGTGGGTGCAGCAA -9824

ATATGACAGCCCAACTGCTGCTGCTGCTGCTAAGTCACTTCAGTCGTGTC -9774

CGACTCTGTGTGACCCCAGAGACGGCAGCCCACCAGGCTCCCCCGTCCCT -9724

GGGATTCTCCAGGCAAGAACACTGGAGTGGGTTGCCATTTCCTTCTCCAA -9674

TGCATGAAAGTGAAAAGTGAAAGTGAAGTCGCTCAGTCGTGTCCGACCCT -9624

CAGTGACCCCATGGACTGCAGCCTACCAGGCTTCTCCGTCCATGGGATTT -9574

TCCAGGCAAGAGTACTGGAGTGGGGTGCCATTGCCTTCTCCATGACAGCC -9524

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CAGTAAAAAGGGCTAAAATAGTGAATCACAGACTTTTGCCAAACAACAGT -9374

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GAATAAAGAAATGTTTGAGCCTGGTTCTCAATGCTTGAATAATAAGATTC -9274

CTTTTCAAGGAAAAACAATTTTTTTGAGAACTCATAGATAAAAGTCAAAT -9224

CTATCACTTTAGATGTCACTTGAAGCCAACTTGATTATAGTAAACAAATT -9174

CAGCTGTAAACATTTTTTTATAACACTGTAGACACTGTATTTTTATACAC -9124

TGTATAAATGAAGACACTGAAGATGTAAAGTGGTGACCTGGATGATCCAG -9074

Fig 1

AATATGCAATATGCACCAAAAACTAAGTTTTTTAAATGATACAAATGAAC -9024

TTACTTACAAAAGGGAAATAGAGTCACAGATGTAGAAAACATACAGTTAC -8974

CTAGGGGGACGGGGGGAGGGTAAACTGGGAGATAGGAATTGACACATACA -8924

CTCTACTATATATACACTAGGTAACTAATATGAACCTACTGTATAGCGCA -8874

CAGAACTCTACCCAATACTCTGTAATGACATTTATGGGAATAGAATCCAA -8824

AAAGAGCAGATATGTGTATATGTATAAACTGATTCACACCCCTTGCCTTG -8774

CCTAAACACACACAGACACACACAGACACATCCATTTTGAGATTTCTTTC -8724

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CTGGGATATTATTATGACACGTTGCCATAATATGAATGTCATCATCTCAA -7774

GACTGACCTGAAAACCAAAATAAAAAAAAAAAGAGAGAGAGAGAGAGA -7724

Fig 1 contd

GAGAGAAAAAAAAGAAAACCCTCCAGACCAGATTTCAGTCTACCACTTGA -7674

GCTGACAAACATTGGCCAAATGTCCTCTACAGAACCTGTAAGTTAGTAGT -7624

TGGTAATTATAAAACAATAAAGTATATATTTCTGCTCCTGGCAATAATTA -7574

TGTATCAGTTAAAAAGTATACATTGTCTAAAAAATCACCATATTTGATGT -7524

CTCATTAAAACAAAGTTACAAATATTAAGATGAGAGGACTTAAAGTTAGA -7474

TGGGAAAATATTCAATTGAAGCAGTATAAAATGCATTACTCTGGGGCAAA -7424

GTGTGGTCTGGAGATCCCTGGAGTGAAGACCCTTTTAGACAATCTGTGGA -7374

GTAAGAACTGTTTTCATAACAAAGCTAAGATTTCCTTGCTATTCTCATTT -7324

TCTCTTACGTATATAGTCGAGTTTTCCAGAAGTTCCATGTTATGTAACAT -7274

CATCATTACTCTGTCAGCAAATAGAATACATGTTTGCATATGTCTATATT -7224

CTAACATTTCTCATTTTTAATTTATAATTCATTAAATATTGATAGATATG -7174

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CATACATGCATCTCTAAATGAAAGCTTTGCCATCTGACTTACTCAAGGTC -6424

ACATAAAACGTCAGCGAGAAACCCAGAACTATATTCCAGATCTCTGTTCC -6374

Fig 1 contd

TATACTGTTACTCCCTGAGTCAAGGGTACTTTTGTTTTTGTTCATTTTTA -6324

TTCTGTAATCTATTGAGATCACAGATAATCAGATGTTGCCACTGTAGGAT -6274

GGCAGCCTCTCATGCTGTTATGTGAATTGAGCACTATCCAGTTTGTTTCT -6224

GGCTTTAAGTGTAATCAGAACAGTGTTATATCAAAGGGCTATCATCACAA -6174

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Fig 1 contd

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Fig 1 contd

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AATATGCTTTATACTCTCAATGTTGTTTTGAAACGCACACCCCCTCCCCT -3574

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TTATGGTTACACAAACGTAAGGCCCCCTGAGTACAAAGAAGAACAGGGGG -3474

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CAGCTGGGATCCTGTGTGTTGGAAGCCTCAAATTTTCCATTGTTCTGTGC -2374

ATACGCACAAATGCTTATAAAAGCACTGTAAGGATTGATTATGAAGTTAA -2324

Fig 1 contd

GATAAATCTCAGCAAGACATAAATGTGCAAGCACGGGATCCATGAATAAC -2274

GAGCACTGACCATGTGGAAATGATAATCTTTGTTTCCTTTATTCCAGGCA -2224

GTAAGGAGAAAGCGCTCACAGGGCTGCCTTACACCATTTTACTAGAGAGC -2174

TAGCCTATGTCAGTCGGTAGCTGGCAATTACAAACTGAAGCAGTTCTAGT -2124

TCATGTGGAGGATGAATTTAACCATAATCTCAACCCCCTCTGCATGAAAC -2074

AGAGACTAAGTACTCAAGTACCAGTTATCAGTCACTTACTATATGACAGG -2024

CACTGTACTCAACAATTTACATGTATTATTGAATTACATGCCCCCAACAC -1974

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TGGCAAACTGAGATCTGAACTCAGGTCTATCCAACTCCAGGACCTGAGAT -1874

CCCAATTGCTACACAATTCTAATCAAGTTAAAAGGGAAAAAGGATTTGAT -1824

TTGCTCAGAAGTGTATAGGGGCATATGTTACAATTATAACATTACAAAGA -1774

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TGAGGCAGTAAATTAGTGTACAATGTGCGAAAGTAGTGACCTAAAAAATA -1474

AATATTTGATATGAACCACTGCACTCTCTTGGGGAAAAAAGTAATGGATT -1424

AACTCTCTTAGGAGTCCTTAGCTTCCCCAAAAGTAGTAGGAAAAATAAAT -1374

CTCCTGTGGCCTGGAAACAGCTTCTGTTTCTTGCTGGCTATATTTGTTTA -1324

GGTTTTTAATAGTT**CATTTG**ATTAGACCTTGTGGCTCCCAAAGCTAAGGT -1274

E-box (4)

TGAGAGTTTGATCCCTACAGAGGCCACTTCAATTTAGAGAACAAAAAGCC -1224

CCATTCTCTGCTCCCAGACCTTACCCCAAATCCCTGCCAGGTGTCTGCCC -1174

TCCGGT**CAAATG**AGAAACTGGCAAAGGAAGTACTAGGAGGTCGCACAGTA -1124

E-box (3)

CTAGGAAGTAGAAAAATGGACTAGCACACTACTGAGAAGCAGAAAAATGG -1074

Fig 1 contd

GCACCCTTCATGATGGTGTTCCTTTCCCTTTCTGTGTTCACAATGCTCCG -1024

ATATAATTTACAGAGGGTAGATAACTACATTTTTTTCTTTTACCACTGGA -974

AGGCTGAGGAAAACTTTGTTACCCATCATAAAATTCACTATCTTCTAAGT -924

CATTCTATGTTATTCTAAGATCAAATAGCTGACAATATCCTCTTTGTAAT -874

AAACAATGAAAAAACACATCCTCTGAGCAATATTAATCTGCAACTTTAGG -824

ATAGGAAGTAACTTAATACTAGTCAATTGAAACTGAAATACAATTTTCAT -774

ATGAATAAAAGATATTATTTAAAAGTAATTCCATGAGCAATTTAATATTA -724

AAGTAGGATTTTCATTATGTGTTAAGAATTTATTCAGGGAAACAAGTTTC -674

TCAAATTATAGCAGAAAATCTTTTACTAGTATCACAGTCTTTTCATTTAA -624

GTCTTCCTGAATAAATCTGTATTTTCTAATTATACAAGA**CTAAAAATAAT** -574

MEF2-site

TTAATATAACAAATAAAATTATTTTTACTT**CAAATG**CTTACTTAAATAG**T** -524

E-box (2)

ATAAAATCATTTTATTTTCTGAGGGAAAAGCATATCAACTTTTTAAGTAT -474

TATA-box (3)

GAAGTGTAAATTAAGATTTATTCACTTAAATTATAATTTTTAAAGTTTCA -424

CATATAAAGATGAATAAGATCTAAGTGTATATGTTATTGTTAATAAAGTT -374

TTTAATTTTTCGCATGTCACATACAGCCTTTATTATTCATAGATTTATTC -324

CTTTTAAGAAGTAGT**CAAATG**AATCAGCTCACCCTTGACTGTAACAAAAT -274

E-box (1)

ACTGTTTGGTGACTTGTGACAGACAGGGTTTTAACCTCTGACAGCGAGAT -224

TCATTGTGGAGCAAGAG**CCAAT**CACAGATCCCGACGACACTTGTCTCATC -174

CAAT-box

AAAGTTGGAA**TATAAAA**AGCCACTTGGAATACAG**TATAAAA**GATTCACTG -124

TATA-box (2) TATA-box (1)

GTGTGGCAAGTTGTCTCTCAGACTGGGCAGGCATTAACGTTTGGCTTGGC -74

GTTACTCAAAAGCAAAAGAAAAGTAAAAGGAAGAAGTAAGAACAAGGGAA -24

Fig 1 contd

AAGATTGTATTGATTTTAAAACCATGCAAAAACTGCAAATCTCTGTTTAT +27

ATTTACCTATTTATGCTGATTGTTGCTGGCCCAGTGGATCTGAATGAGAA +77

CAGCGAGCAGAAG +90

FIG. 1. Nucleotide sequence of the 10,492-basepair promoter-enhancer region of bovine myostatin. Total length of the DNA sequence is 10,492 basepairs (bps) which consist of 10,402 bps of upstream and 90 bps of coding sequence. The coding sequence of myostatin is underlined. Nucleotides and the relative position of putative nucleotide motifs are numbered with respect to the translation start site (+1 bp).

Consensus sequences for basic functional elements of a mammalian promoter (TATA and CAAT boxes) and for known muscle specific transcription factors (E-box and MEF2 sites) are bolded in the sequence and they are listed below.

TATA-boxes[1]:	-139 bps(1); -163 bps(2);
	-524 bps(3)
CAAT-box[2]:	-206 bps
E-boxes[3]:	-308 bps(1); -543 bps(2)
	-1167 bps(3); -1309 bps(4)
MEF2 site[4]:	-584 bps

Fig 1 contd

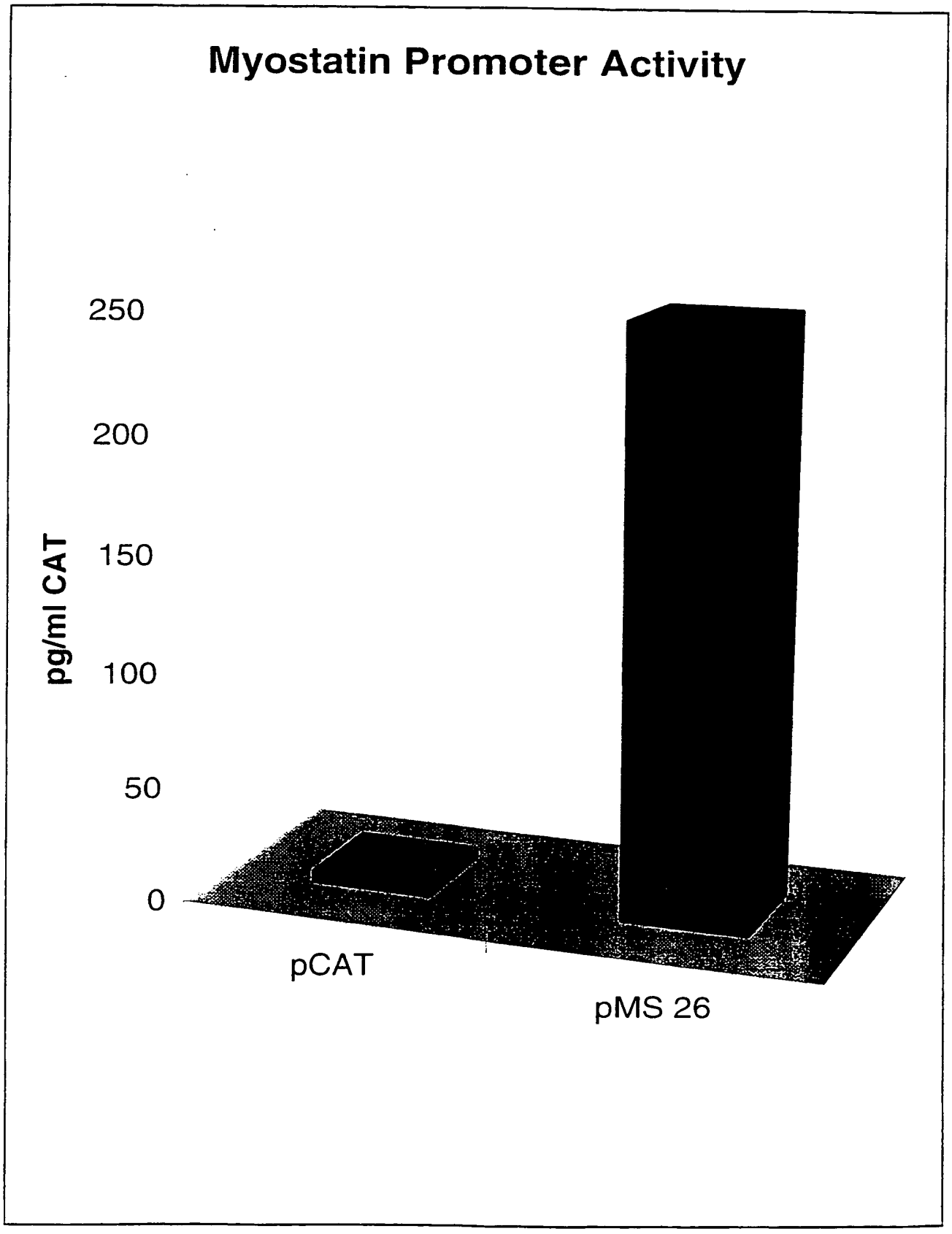
Myostatin Promoter Activity
250
200
150
100
50
0
pg/ml CAT
pCAT
pMS 26

Fig 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 99/00107

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 015/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
As above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, Medline: - ((Myostatin or GDF8) and promoter))
GenBank. EMBL, TRMBL, PIR: -Nucleotide sequence

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Acc No AF093798. Sus scrofa myostatin (GDF8) gene, promoter region and partial cds.	1-15
A	McPherson AC and Lee S-J. Double muscling in cattle due to mutations in the myostatin gene.	All

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1999	- 6 OCT 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **GILLIAN ALLEN** Telephone No.: (02) 6283 2266